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Protein aggregates modulate the texture of emulsified and acidified acid milk gels

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Abstract

Acid milk gels with fat (3.5 or 10%) or without fat containing 0.2 to 1% of whey proteins (WP) or 0.2 to 1.5% of protein aggregates (fractal aggregates, microgels and mixed casein/WP aggregates with an 80/20 ratio) were investigated. The fat-containing systems were homogenized and the systems were preheated (90°C, 11 min) and acidified to a pH of 4.6 by adding glucono-delta-lactone. The protein composition of the fat droplet interface was characterized by SDS-PAGE and the textural and rheological properties, microstructure, and whey separation of acid networks were determined. WP and fractal aggregates showed the best ability to improve the textural properties and microstructure of all acid milk gels and reduce whey separation. Increasing the concentrations in microgels and mixed aggregates did not lead to an increase in gel firmness or generate a strong impact on the protein network, but a high concentration in mixed aggregates could reduce whey separation. The fat droplet interface was made almost exclusively of caseins, even if the fractal aggregates could also be adsorbed when the interfacial surface was increased either through fat content or through an increase in homogenization pressure. Adding proteins changes the textural properties of the acid gels in all the systems, mainly due to their role in the continuous phase.

Keywords

Protein aggregates, texture, acid milk gels, rheological measurements, whey separation, protein network, interfacial composition

Highlights

- WP addition is the most efficient way to improve texture in acid milk gel.
- Fractal aggregates led to firmer and more solid-like gels with less wheying-off.
- Mixed and microgel aggregates formed protein enriched gels with stable firmness.
- Increased interfacial surface led to fractal aggregates-enriched interfaces.

Chemical compounds used in this article:

Sodium Dodecyl Sulfate (PubChem CID: 3423265); Sodium Azide (PubChem CID: 33557); Nile red (PubChem CID: 65182); Alexa fluor 488 (PubChem CID: 102384756).

1- Introduction

The quality of fermented dairy products such as yogurts depends on a fairly complex assembly of criteria to be appreciated by consumers such as their texture, taste and health benefits (Vasbinder, Alting, Visschers, & De Kruif, 2003). How texture is perceived has a strong impact on product acceptance and sensory qualities. Texture depends on the composition of the product (protein concentration, fat content...), but also on how it is produced. Milk homogenization at different pressures is conducted to prevent fat separation, to decrease the size of fat droplets and reduce whey separation (Lucey & Singh, 1997). A large portion of whey proteins are denatured by the heat treatment also used to eliminate the potential presence of pathogenic microorganisms (Guyomarc'h, Jemin, Tilly, Madec, & Famelart, 2009; Lucey, Munro, & Singh, 1998; Lucey, Teo, Munro, & Singh, 1998).

Heating milk at a temperature exceeding 80 °C increases gel firmness. However, the effect of this parameter on the milk and on the acid gels strongly depends on both temperature and duration: heat treatments at very high temperatures, such as sterilization (110 °C for 30 min or 130 °C for

45 s) or UHT treatment (145 °C for 1 to 2 s) can change the flavor and color of milk due to a Maillard reaction (Sfakianakis & Tzia, 2014). Heat treatments applied to milk induce the denaturation of whey proteins (WP) at temperatures between 60 and 80 °C (Anema & Li, 2015; Cayot & Lorient, 1998; Chen & Dickinson, 1998; Paulsson & Dejmek, 1990). During the denaturation step, the low energy bonds that stabilize the secondary and tertiary structures of proteins are broken (hydrogen bonds, hydrophobic or Van der Waals interactions). The hydrophobic residues, present in the core of the native globular structure get exposed at the surface (Cayot & Lorient, 1998; Kella & Kinsella, 1988). As a result, at a concentration of proteins which is sufficient, hydrophobic interactions and disulfide bonds between the denatured proteins lead to the formation of aggregates. Denatured whey proteins interact with each other, or with the κ -casein at the surface of the casein micelle, via disulfide bridges and low energy bonds. These modifies the initial properties of proteins, such as their hydrophobicity and charge (Donato & Guyomarc'h, 2009; Mahmoudi, Axelos, & Riaublanc, 2011; Mahmoudi, Gaillard, Boué, Axelos, & Riaublanc, 2010; Vasbinder, Van Mil, Bot, & De Kruif, 2001). The interactions of whey proteins with caseins increase the hydrophobicity of the micellar surface, which has been reported to promote gelation (Lucey, Munro, et al., 1998). It also leads to an increase in gel firmness (Guyomarc'h et al., 2009; Lucey & Singh, 1997; Vasbinder et al., 2001). Furthermore, newly formed soluble aggregates can be trapped in the protein network or remain present in the aqueous phase. Heat treatment was shown to also increase whey retention capability (Guyomarc'h et al., 2009; Lucey, Munro, et al., 1998; Lucey & Singh, 1997; Lucey, Teo, et al., 1998; Vasbinder et al., 2001). Altogether, these contribute to the modification of the rheological properties of the final products and it has been a major concern for the optimization of the texture of yoghurt. The effects of heat treatment and of the addition of WP on the rheological properties of acid milk gels have already been studied (Lucey, Munro, & Singh, 1999; Lucey, Teo, Munro, & Singh, 1997) and show a strong increase in gel firmness. When adding different types of whey protein aggregates of different sizes, it was shown that the microstructural properties of acid milk gels without fat can be improved with small aggregates (Liu et al., 2016). Moreover, gels containing WP or WP aggregates reach higher G' values and gel earlier. This may be due to a greater reactivity of the -SH groups of whey proteins after denaturation, and to their interactions with caseins (Aguilera, Xiong, & Kinsella, 1993; Morand, Guyomarc'h, Pezennec, & Famelart, 2011).

During milk acidification - carried out either with glucono delta lactone (GDL) or with lactic acid bacteria - the pH gradually decreases. This leads to physicochemical modifications of the casein micelles through, for instance, i) the progressive cancellation of negative charges of caseins due to the protonation of carboxylic acid functions and ii) the solubilization of minerals, such as colloidal calcium phosphate, that were stabilizing the micellar structure (Walstra, 1990). When the pH reaches 4.6, the electrostatic repulsions are cancelled and hydrophobic and hydrogen interactions become preponderant, allowing the caseins to form a network. If their concentration is large enough, a firm gel is finally formed (Anema & Li, 2000; Famelart, Lapesant, Gaucheron, Le Graet, & Schuck, 1996; Le Graet & Brulé, 1993; Lucey & Singh, 1997; Vasbinder et al., 2001).

Due to denaturation and aggregation, different types of aggregates can be produced when heating a whey protein solution, depending on the pH of the solution, the ionic strength and the protein concentration (Jung, Savin, Pouzot, Schmitt, & Mezzenga, 2008; Nicolai, Britten, & Schmitt, 2011). When the pH reaches the isoelectric point, the proteins are weakly charged and will easily aggregate in order to form dense and spherical aggregates called microgels (Donato, Schmitt, Bovetto, & Rouvet, 2009). Microgel aggregates can also be obtained at a pH close to neutral in the presence of calcium (Phan-Xuan et al., 2014). When the pH is close to 7, whey proteins are negatively charged and fractal aggregates can be obtained by heat treatment (Nicolai, Britten, & Schmitt, 2011). Under this pH condition, the addition of NaCl leads to a decrease of the electrostatic repulsions. This results in the formation of large and dense fractal aggregates (Mahmoudi, Mehalebi, Nicolai, Durand, & Riaublanc, 2007). A final type of aggregates can be obtained by heating a WP solution in the presence of casein micelles at a pH of 6.3. In this case, the denatured whey proteins are known to bind to the micelle surface to form what is generally called mixed aggregates. The fine ratio of caseins and whey proteins contained in these mixed aggregates have been determined in former studies (Anema & Li, 2000, 2003; Loiseleux et al., 2018). Whereas the impact of native whey proteins on gel firmness has been extensively studied, it has not been clearly defined how pre-aggregates of different morphologies affect the formation of gel. The nature of protein aggregate is supposed to influence the interactions with the network of casein or to interact with the interface of fat droplet. This should modulate the rheological properties of acid milk gels. Torres et al. (2018) already demonstrated that a high content of native whey proteins associated with microparticulated whey proteins considerably increased the

viscosity, elasticity and resistance of stirred yoghurts. Microparticles behave like active fillers in the network of proteins and decrease whey separation.

The texture of an acid gel prepared from milk is also related to its fat content and to the size of the fat droplets. Increasing the fat content increases the strength of acid milk gels and decreases the duration to form a gel (Aguilera et al., 1993). This has been shown to result from the interactions between caseins and the proteins present at the surface of fat droplets (Aguilera & Kinsella, 1991). Moreover, increasing the pressure for the homogenization decreases the size of fat droplets (Jost, Baechler, & Masson, 1986). Other parameters alter the size of fat droplets such as the concentration of emulsifiers and the nature of proteins (Dickinson, 2003). For example, as native whey proteins have a high ability to cover large surface, their addition to model o/w emulsions produces fat droplets with smaller size than those obtained when whey protein aggregates (WPA) are added (Kiokias & Bot, 2006).

Whey separation is a common defect in yoghurts. It can occur especially if the gel network is damaged or if the gel undergoes a major structural reorganization (Lucey & Singh, 1997). Spontaneous whey separation can be defined as shrinking of the gel without application of external forces. It is related to the instability of the gel network that cannot trap the entire liquid phase anymore. Whey separation can be reduced, for example, by increasing the total solid content. There are two ways of acting on this parameter: increasing fat or protein concentrations (Delikanli & Ozcan, 2017; Isleten & Karagul-Yuceer, 2006; Keogh & O’Kennedy, 1998). Adding increased amounts of proteins before acidification results in a decrease of porosity in the gel. The protein network becomes denser and the protein material is better distributed inside the gel. This improves the homogeneity of the gel structure (Andoyo, Guyomarc’h, Burel, & Famelart, 2015; Andoyo, Guyomarc’h, Cauty, & Famelart, 2014). The presence of fat leads to more connections between proteins in the continuous phase (residual native whey protein or soluble and insoluble aggregates) and proteins localized at the interface, leading to a decrease in whey separation (Keogh & O’Kennedy, 1998).

As shown before, the effect of adding whey protein in milk before acidification on the final gel properties is better known than the one of protein aggregates. The aim of this study is to determine how adding different WPA can alter the rheological properties of acid milk gels (with or without fat in their content), and how WPA reduce whey separation. Different concentrations

and types of protein aggregates were studied, together with different homogenization pressures and fat contents.

2- Materials and methods

2.1- Raw materials

Anhydrous milk fat TREX 32C (melting point 32 °C) was kindly provided by Corman (Limbourg, Belgium). Whey protein isolate (WPI), casein powder (Cas), milk permeate powder and milk powder were supplied by local dairy companies (confidential origin).

Low heat WPI powder was used as the source of purified whey proteins. It was obtained as described by Chevallier et al. (2018). Briefly, milk microfiltrated by ultrafiltration and diafiltration was used to isolate a whey protein concentrate. The concentrate was then spray dried. This powder contained 91.1% (w/w) proteins - determined by the Kjeldahl method -, 5.21% moisture, $\leq 3\%$ lactose, $< 3\%$ mineral and $\leq 0.4\%$ fat. The protein content of the WPI powder was composed of 12% of caseins and 88% of whey proteins. Cas was composed of a small fraction of whey proteins (6.6%) and 81.7% (w/w) of caseins which were mostly in a micellar state (Loiseleux et al., 2018). This powder contained $\leq 6\%$ moisture, 4% lactose, 4% mineral and $\leq 2\%$ fat. Milk permeate powder was produced through ultrafiltration of skimmed milk using a membrane (molecular weight cut-off of 10 kDa) and was used in this study as a source of milk-soluble minerals. This powder contained $< 4\%$ moisture, $> 80\%$ lactose, 4% protein and $\leq 1\%$ fat. The mineral content of the milk permeate powder determined by atomic adsorption was composed as following: Ca^{2+} : 0.31%; Mg^{2+} : 0.12%; Na^{+} : 0.63%; K^{+} : 2.74%. The milk powder was obtained from a skimmed milk obtained by atomization and having undergone a minimum heat treatment. The casein/whey protein ratio, given by the supplier, was close to 80/20. The composition of this milk powder is given in Table 1.

2.2- Production of aggregates and recombined milk preparation

The protein aggregates (fractal, microgel and mixed aggregates) were produced according to the protocols used by Loiseleux et al. (2018) which are briefly summarized below. Once produced, the aggregates were cooled down at room temperature and stored at 4 °C for a maximum of 1 month. To avoid microbial development, sodium azide at 0.2 g.L⁻¹ was added to all heated

solutions. It was confirmed by dynamic light scattering measurements that aggregate morphologies did not vary during storage (see 2.3.1).

2.2.1- Fractal aggregates

WPI powder was dissolved overnight under magnetic stirring at 4 °C in milli-Q water (200 mL) at a concentration of approximately 80 g.L⁻¹. Protein concentration was checked using a UV 1800-spectrophotometer (Shimadzu, Kyoto, Japan) by absorption measurement at 278 nm using an extinction coefficient of 1.046 L.mol⁻¹.cm⁻¹ (Mahmoudi et al., 2007). The solution was diluted to 50 g.L⁻¹ using milli-Q water. NaCl (final concentration: 0 to 45 mM) was added in order to obtain different sizes of fractal aggregates. The pH was adjusted to 7 with sodium hydroxide. A volume of 200 mL was subsequently heated at 80 °C for 2 h without stirring in order to produce fractal aggregates.

2.2.2- Microgel aggregates

The WPI solution was prepared at 50 g.L⁻¹ as previously described. The pH was adjusted to 5.8 with hydrochloric acid. A volume of 200 mL was heated at 85 °C for 1 h without stirring in order to produce microgel aggregates.

2.2.3- Mixed aggregates

To be close to the mineral composition of milk and maintain the integrity of the casein micelles, an aqueous solution of milk permeate was used as a solvent. On the one hand, 100 mL of a 50 g.L⁻¹ solution of whey proteins (WP) were prepared in a 56 g.L⁻¹ aqueous milk permeate solution and were left under stirring overnight at 4 °C. On the other hand, 200 mL of a 50 g.L⁻¹ casein dispersion (Cas) were also prepared in the same milk permeate solution under stirring at 20 °C for 30 min. Hydrochloric acid (6 M) was added to the Cas dispersion in order to reach a pH of 6.3. The dispersion was maintained under magnetic stirring during 45 min at 60 °C, and then overnight at 20 °C to correctly rehydrate the powder. The WP solution and the Cas dispersion were mixed in order to obtain a 80/20 Cas/WP ratio (matching the milk ratio) at a total protein concentration of 50 g.L⁻¹. The pH of the mix was adjusted to 6.3 with hydrochloric acid (6 M) and the solution was heated at 80 °C for 1 h without stirring.

2.2.4- Preparation of recombined milk

The milk was reconstituted by reincorporating skimmed milk powder in a volume of Milli-Q water under magnetic stirring (500 rpm for 1.5 h) at room temperature to obtain a final protein concentration of 35 g.L⁻¹. 2 to 15 g.L⁻¹ of native whey proteins or protein aggregates were added to the milk with a stabilization time of 15 min. The recombined milk that did not contain WP or protein aggregates was named “control milk”.

Systems with or without fat were studied. In the first case, anhydrous milk fat (AMF) and milk were heated at 60 °C before mixing. Homogenization was then carried out on the mixture (3.5% or 10% AMF) in two consecutive stages. A volume of 300 mL of sample was pre-emulsificated with a rotor-stator homogenizer SilentCrusher M (Heidolph, Germany) for 3 min at 14,000 rpm with a 12 mm head. Then, a high-pressure homogenizer Panda plus 1000 (GEA Niro Soavi, Italy) was used to reduce the size of the fat droplets, through recirculation at 100 or 500 bars at 29 L/min for 5 min (with an average of 6 cycles). 50 mL of sample were heated under magnetic stirring at 90 °C for 11 min (including the temperature rise). It was then cooled down with ice for 15 min, and stirred at 40 °C for 5 min. The residual NWP content was determined after the heat treatment of recombined milk. It was of 6 g/L and this value was constant for all the samples. Glucono- δ -lactone (GDL) at a pre-determined concentration, depending on the protein and the fat contents, was added to the systems in order to acidify them at a pH value of about 4.6 after 6 h (1.1% -1.3% (w/w)). The experimental steps of recombined milk preparation with fat are presented in Figure 1.

In absence of fat, the heat treatment was directly performed on 50 mL of sample after adding proteins to the reconstituted milk. A GDL concentration within the same range as for the recombined milk with fat was added.

2.3- Aggregate characterization

2.3.1- Size distribution of aggregates

A Zetasizer Nano Series (Malvern Instrument, UK) was used to determine the size (Z-average hydrodynamic diameter) and the size distribution of the aggregates after the heat treatment with dynamic light scattering (DLS). The aggregate solutions were diluted 100 times with Milli-Q water. The diluted solutions were placed in a plastic cell and analyzed at 20 °C in a backscattering configuration at 173° for 120 s. The hydrodynamic diameter was measured in

triplicate. A refractive index of 1.45 was used for protein particles (Zhang, Arrighi, Campbell, Lonchamp, & Euston, 2016) in order to calculate the volume-size distribution.

2.3.2- Transmission Electron Microscopy (TEM)

Aggregate solutions were diluted 500 times in Milli-Q water. A volume of 15 μ L of diluted sample was deposited on a carbon grid (200 meshes). After 2 min, the excess was removed with an absorbent paper. The samples were stained with 2% of uranyl acetate solution for 2 min before removing the excess, and then dried for 15 min at 60 °C. The aggregates were observed using a transmission electron microscope JEM-1230 (JEOL, Tokyo, Japan) operating at 80 kV.

2.4- Analyzes of milk emulsions

2.4.1- Fat droplet size determination

The fat droplet size distribution in emulsions was determined directly after emulsification using a laser light scattering instrument (Partica LA-960, Horiba, Kyoto, Japan), and at least one hour after a tenfold dilution by 1 % (w/v) of sodium dodecyl sulfate (SDS), in order to respectively obtain the size of the flocs and of the individual droplets. Size distribution was estimated using the Mie theory (Mie, 1908) and a refractive index of 1.458 for the dispersed phase (anhydrous milk fat) and 1.333 for the continuous phase (water). The mean diameter of the fat droplets was evaluated by the volume average diameter (D_{43}).

2.4.2- Determination of the interfacial composition

Proteins were recovered from the fat droplet surface using the Patton & Huston (1986) method modified by Surel et al. (2014). The studied systems contained 3.5% or 10% (w/w) of AMF (to increase the interfacial surface) and 1% (w/w) of WP or aggregates. This analyze was performed on the milk after the homogenization step at 100 bars and after the heat treatment, but before gelation. This technique makes possible to separate the fat droplets from the emulsions in a single centrifugation step. 2 mL of emulsion were diluted in 2 mL of 50% (w/v) saccharose solution in order to increase the density of the aqueous phase. It was then slowly deposited at the bottom of a 15 mL-centrifuge tube under 10 mL of a 5% (w/v) saccharose solution. The tube was then subjected to moderate centrifugation (3,000 g for 2 h at 20 °C) which caused creaming of the droplets. The droplets passed through the 5% (w/v) saccharose solution and were washed from all

unabsorbed constituents. After centrifugation, three phases were obtained: the top one (1) with the cream containing the washed fat droplets, the intermediate phase (2) with the 5% (w/v) saccharose solution, and the bottom one (3) with the aqueous phase of the emulsion containing the unabsorbed constituents.

The tubes were then immediately frozen at -20 °C in a vertical position to avoid redispersion of the different phases. They were then cut to recover each phase separately and study their composition. A prior desorption of proteins adsorbed at the interface was necessary. The top part (1) was recovered, weighed and diluted to 1/3 in a solution of 1% SDS. The mixture was vortexed and left standing for 1 h at 40 °C. It was then centrifuged at 3,000 g for 1 h at 10 °C. Two phases were obtained, the top phase (4) containing fat droplets stabilized by SDS and the bottom phase (5) containing the solution of desorbed proteins. The bottom phase (5) was recovered using a syringe, then diluted in a denaturing solution containing 8 M of urea, 5 g/L of SDS, 0.2 M of tris buffer, 0.05 M of trisodium citrate and 10 mM of β -mercaptoethanol (β Me). The aqueous phase (3) after thawing was also diluted in this denaturing solution. For phases 3 and 5, the protein concentration was quantified by UV absorption at 278 nm using a 1800-spectrophotometer (Shimadzu, Kyoto, Japan) for which extinction coefficient was set up to 1.046 L.mol⁻¹.cm⁻¹ (Mahmoudi et al., 2007). The protein composition of the milk and of phases 3 and 5 was determined by SDS-PAGE.

2.4.3- Protein composition

The proteins adsorbed onto the fat droplet surface of the milk (phase 5), with or without added whey proteins or aggregates, were identified by SDS-PAGE. The solubilization buffer was prepared in a reducing condition (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β Me, 0.1% bromophenol blue) or non-reducing condition (same composition without β Me). Phases 3 and 5 were diluted to a concentration of 2 mg.mL⁻¹ and a volume of this solution was again diluted in one volume of solubilization buffer. The samples were then placed for 5 min at 100 °C in order to denature the proteins.

SDS-PAGE were performed using 10-well precast gels with a polyacrylamide concentration ranging from 4 to 12% (Bolt™ 4-12% Bis-Tris Plus, Invitrogen, Thermo Fisher Scientific, Waltham, United-States). In order to estimate the molecular weight of the proteins, a molecular

standard (Novex® Sharp Pre-Stained Protein Standard, Invitrogen, Thermo Fisher Scientific, Waltham, United States of America) was deposited in the first well. In each well, 10 µL of standard or 15 µL of sample were deposited. The gels were placed in the migration vessel containing the MES-SDS 1X (Bolt MES SDS running buffer (20X), Novex, Life Technology, Carlsbad, United States of America) and electrophoresis was carried out at constant current (60 mA) for 1.5 h with a power source generator at 300 V (VWR, Radnor, United States of America). The gels were stained with Instant Blue™ (Expedeon, Cambridgeshire, United Kingdom) and scanned to identify proteins.

2.5- Acid gelation characterization

2.5.1- Rheological measurements of acid milk gels

Rheological experiments were performed using a AR 2000 controlled stress rheometer (TA Instruments, Leatherhead, United Kingdom) equipped with a Couette geometry (stator radius: 15 mm; rotor radius: 14 mm; cylinder immersed height: 42 mm) thermostated by a Peltier module. The sample was deposited at 40 °C in the outer cylinder just after the addition of GDL (t_0). The inner cylinder was lowered until it was completely immersed in the sample, and then the pH electrode (Mettler Toledo InLab® Micro, Mettler Toledo, Schwerzenbach, Switzerland) was placed in the sample above the Couette geometry for *in situ* measurements. The sample was then covered with paraffin oil to prevent evaporation.

A time sweep was first done to follow the changes in G' and G'' during 6 h of acidification (1 rad/s, strain 1%) at 40 °C while the pH was measured. When the pH reached 4.6, a frequency sweep (0.01 to 100 rad/s, strain 1%) was performed at a constant temperature of 40 °C, followed by a strain sweep (0.02 to 4%, 1 rad/s, 40 °C) to ensure that the measurements were done in the linear viscoelastic region. The measurements were performed at least twice. Time and pH of gelation, as well as final G' were determined from the acidification kinetics. The change of loss tangent ($\tan \delta = G''/G'$) versus time and pH were analyzed in order to obtain the gelation time and pH from the asymptote of $\tan \delta$ (Lucey, Munro, et al., 1998; Lucey, Teo, et al., 1998). The amplitude of the loss tangent at gelation pH was also determined. The G' slope was determined from the frequency sweep and gave information on the structure and the strength of the gels formed at pH 4.6.

2.5.2- Confocal Laser Scanning Microscopy (CLSM)

A NIKON Eclipse-TE2000-A1si (Champigny sur Marne, France) confocal laser scanning microscope was used to characterize the microstructure of gels containing 3.5 % (w/w) of fat and 1 % (w/w) of WP or aggregates. Immediately after the GDL addition, a droplet of milk sample was deposited on a microscope slide equipped with a spacer that has a depth of 250 µm. Several microliters of two dyes, Nile Red (for lipids) dissolved in dimethylformamide (0.5 µg/µL) and Alexa 488 (for proteins) dissolved in methanol (0.2 µg/µL), were added to the sample and mixed. The system was sealed with a cover glass to prevent evaporation. The samples were covered with aluminum foil - to protect the fluorescent probes from light until sample analysis - and placed in an oven maintained at 40 °C for 6 h. Samples with a pH of 4.6 were observed using a x20 lens (Plan APO 20X; numerical aperture: 0.75) and a water immersion lens x40 (Plan APO 40X; numerical aperture: 1.25) using an Argon laser (corresponding to excitation of the Alexa 488) and the 561 nm Laser Diode (Nile Red).

2.5.3- Whey separation analysis

Whey separation was determined according to the centrifugation method (Keogh & O’Kennedy, 1998) with some modifications. Immediately after stirring, 10 mL of sample with GDL were placed in three previously weighed tube and stored at 40 °C for 6 h, and then at 4 °C for one night (D1), 7 days (D7) or 14 days (D14). Centrifugation at 4 °C and 3,500 rpm for 15 min were performed at D1, D7 and D14. After 1 min of incubation without stirring, the supernatant was recovered and weighed. Whey separation was calculated according to the following equation (Keogh & O’Kennedy, 1998):

$$\text{Whey separation (\%)} = (\text{Weight of supernatant (g)} / \text{Weight of gel sample (g)}) \times 100$$

2.6- Statistical analyses

The presented results were obtained from at least two repetitions of complete sample preparation and analysis. ANOVA was performed using StatGraphics ® Plus, 5.1 (Statpoint Technologies, Inc., Warrenton, VA, USA) with a Tukey test (95.0%). Statistical analyses were performed on rheological analysis on control acid milk gels with or without fat, on acid milk gels with 1% added WP, and fractal, microgel or mixed aggregates with or without fat. These statistical

analyses were also performed when studying whey separation, the effect of the size of fractal aggregates and the one of homogenization pressure.

3- Results and discussion

3.1- Aggregate characterization

3.1.1- Fractal aggregates

The Z-average diameter of fractal aggregates produced at 80 °C during 2 h at 45, 20 and 0 mM of added sodium chloride was respectively 228, 93 nm and 68 nm (Figure 2A). Depending on their size, the fractal aggregates were called Fa228, Fa93 and Fa68. Observations made by TEM evidenced a more branched structure for Fa228 than the ones produced at the lowest NaCl content (Fa93 and Fa68) (Figure 2B). This reflects a higher size polydispersity for the aggregates present in Fa228 by comparison to those present in Fa93 and Fa68. TEM observations show Fa228 fractal aggregates forms cluster and over-aggregates. Consequently, the structures and the size of fractal aggregates could be monitored according to the ionic strength of the solution.

3.1.2- Microgel aggregates

Microgel aggregates exhibited a Z-average diameter of 450 nm (Figure 3A). They appeared in the TEM as dense, circular and were mostly grouped (Figure 3B). These observations explain the results obtained by dynamic light scattering (DLS) measurement. Individual microgel aggregates exhibited a diameter of 150-200 nm.

3.1.3- Mixed aggregates

The size distribution of mixed aggregates could not be obtained using DLS, because it was not possible to discriminate between casein micelles and mixed aggregates (Loiseleux et al., 2018). Mixed aggregates observed by TEM (Figure 4) exhibited, as expected, a core composed of a casein micelle (average diameter between 100 and 200 nm) and a surface of whey protein aggregates that were more or less branched.

3.2- Gelation properties of acid milk gels with or without fat and protein aggregates

3.2.1- Control acid milk gels with or without anhydrous milk fat (AMF)

First experiments were made on control acid milk gels, namely without further addition of proteins, with or without 3.5% of AMF. For systems with AMF, homogenization at 100 bars resulted in fat droplets of a mean diameter of 0.8 μm (D_{43}).

The average results obtained for acid milk gels are presented in Table 2. Similar time (around 29 min) and pH of gelation (5.45) were obtained independently of the presence of fat. However, compared to control acid gel without fat, fatty gels had a higher final storage modulus from over 100 Pa. The G' slope obtained from the frequency sweep of the AMF gels was lower (0.150) than without AMF (0.164). This showed that the addition of anhydrous milk fat allowed the formation of stronger and more structured systems, in accordance with the results obtained by Aguilera et al. (1993).

3.2.2- Impact of native whey protein addition on acid milk gel properties

In the same way, the gelation of acidified milk with or without fat in the presence of variable concentrations of native whey proteins added before heat treatment was also monitored. A linear increase of final G' with native whey protein (NWP) concentration added was obtained with or without fat. The values obtained for acid milk gels with AMF were of course higher (Figure 5A). Furthermore, for acid gels containing 1% added NWP, a strong decrease in gelation time and therefore an increase in gelation pH were obtained (Table 2). It has already been established that increasing whey protein concentration in milk before heat treatment induces a better structuring and higher firmness of acid gels, and a decrease of the gelation time (Lucey & Singh, 1997). The heat treatment applied on the milk induces the denaturation of whey proteins (Anema & Li, 2015; Cayot & Lorient, 1998; Chen & Dickinson, 1998; Paulsson & Dejmek, 1990) and their interactions with each other or with κ -casein at the surface of the casein micelles. Whey protein aggregates formed when heating the gel at a pH of about 5.6, while pure casein micelles formed a gel of a pH of approximately 5.0 (Vasbinder, de Velde, & de Kruif, 2004). The higher gelation pH of whey protein aggregates can be explained by their pI values and also by their significantly higher surface hydrophobicity (Morand, Dekkari, Guyomarc'h, & Famelart, 2012; Morand, Guyomarc'h, Legland, & Famelart, 2012). During the acidification process, the WP aggregates were shown to interact with casein micelles and accelerate their destabilization. It causes both a decrease in gelation time and an increase in gel firmness (Andoyo et al., 2015; Ozcan, Horne, & Lucey, 2015).

Furthermore, compared to the control, a decrease of $\tan \delta$ amplitude and G' slope from the frequency sweep was also obtained with 1% of WP regardless of the presence of AMF (Table 2). All these observations confirmed that a more structured protein network, with more connections or stronger bonds, was produced in the presence of a higher WP concentration. Lucey & Singh (1997) and van Vliet, van Dijk, Zoon, & Walstra (1991) considered that the $\tan \delta$ amplitude is related to the possible relaxation of bonds during the initial period after the formation of gel. Less relaxation (lower $\tan \delta$ amplitude) can be explained by a more solid-like system. It is noteworthy to mention that similar changes in G' were obtained with or without AMF. In both cases, G' increased along with the NWP concentration. This can be seen in Figure 5A where the two lines are nearly parallel. By comparison with gel without AMF, the final G' of all gels containing AMF were higher. The increase of G' may evidence that fat concentrates proteins in the continuous phase by steric hindrance and that the interaction between the network of casein and the proteins coated at the interface of fat droplets has been improved. Increasing the fat concentration thus increases the strength of acid milk gels and shortens the gelation time (Aguilera & Kinsella, 1988, 1991; Aguilera et al., 1993). Fat droplets covered with proteins can then act as a nuclei on which native or aggregated whey proteins and casein micelles connect during gel formation (Jost, Dannenberg, & Rosset, 1989). In parallel, Aguilera & Kinsella (1988, 1991) have also shown that the addition of WP to milk with fat improved the rheological properties of acid gels. In our case, the changes observed upon addition of NWP were similar regardless of the presence of AMF. It indicates that whey proteins mostly play a role in the main protein network and are weakly or not involved in the interactions at the interface. The presence of fat thus reinforces the protein network mainly by steric exclusion.

The results obtained for the separation of whey in fat systems are presented in Figure 5B. Separation of whey close to 0% was obtained for acid gels at 0.4 to 1% WP and was stable over time, while systems with no addition of whey protein showed a large separation of whey (between 10 and 15%). By comparison, the value obtained for systems without fat at 1% WP added was around 50%. This result is in agreement with the literature, Keogh & O'Kennedy (1998) having shown that fat decreased whey separation. The presence of fat together with a higher concentration of proteins increased the ability of the reinforced protein network to immobilize the aqueous phase. Furthermore, it has also been established that increasing protein concentration reduced whey separation (Delikanli & Ozcan, 2017; Isleten & Karagul-Yuceer,

2006), and nonfat yogurts fortified with WPI had a lower level of whey separation than without addition of WPI (Isleten & Karagul-Yuceer, 2006).

3.2.3- Impact of protein aggregate addition on acid milk gelation

Acid milk gels with or without 3.5% AMF and with 0.2 to 1% of fractal aggregates (Fa228) as well as 0.6 to 1.5% of microgels or mixed aggregates were produced. For microgel and mixed aggregates, the lowest concentrations (0.2 and 0.4%) were not tested, as analyses on systems without fat did not show any changes in the final G' and whey separation compared to control acid milk gels. The results for acid gelation of 3.5% AMF systems containing protein aggregates are reported in Figure 6 and Table 2.

In presence of 1% Fa228, a decrease in gelation time related to an increase in gelation pH was observed with or without AMF compared to the control, as it is the case in systems with WP (Table 2). Furthermore, the addition of increasing amounts of fractal aggregates also led to a linear increase of G' (Figure 6). Additionally, as it was the case with acid gels without fat, the G' values were lower than those obtained when adding NWP. Addition of these fractal aggregates also led to both a lower G' slope and $\tan \delta$ value. Although G' was lower, indicating less connections, these two last parameters indicated a better structuration of the gel, probably because of stronger interactions between the fractal aggregates and the casein network. The whey separation analysis showed the same changes than when adding NWP, in the presence and the absence of added fat (Figure 7). Whey proteins and fractal aggregates were both able to participate in the formation and the strengthening of the protein network, without strong interactions with the fat droplet interface.

By contrast, as shown in Table 2, adding microgel aggregates (1%) did not affect the final G' value of acid milk gels with or without AMF compared to the control with or without AMF. Furthermore, as shown in Figure 6, the final storage moduli after 6 h of acidification were stable for gels with AMF and 0.6 to 1.5% of microgel aggregates. However, the rheological analyses showed a significant increase in gelation time and a decrease in gelation pH compared to controls (shown for a concentration of 1% in Table 2). This means that, by contrast with the addition of WP and fractal aggregates, preformed microgels did not establish interactions with any component of the system and were unable to strengthen the texture. It may be due to their larger

size and higher density compared to fractal structures. Microgel aggregates were less numerous than WP or fractal aggregates and they seemed to have an inert role in the network. These results are in agreement with those obtained by Liu et al. (2016), who have shown that aggregates of smaller sizes were more effective for texturing acid milk gels without fat. They explained that microparticulated aggregates, unlike nanoparticulate aggregates, did not form strong structures via disulphide interactions with proteins in milk without fat during heat treatment and were also unable to become part of the protein network. During acidification, larger aggregates may restrict bonding with both caseins and other whey proteins, so the protein network was less structured. These results were different from those reported by Torres et al. (2018), showing an increase of the texture of yoghurts upon addition of such aggregates. This was ascribed to an active filling of the aggregates, establishing connections with the main protein network. The authors pointed out that those residual native whey proteins were included in their aggregate samples, explaining their higher functional effect after heat treatment. In our case, very few NWP were present in the samples (Loiseleux et al., 2018) and increasing microgel aggregate amounts did not lead to change the rheological properties. Furthermore, the aggregates used in this study are produced at a higher temperature than the ones used by Torres et al. (2018). This parameter potentially was the origin of the differences in functionality. In addition, the whey separation obtained on acid milk gels with AMF and with addition of 1.5% of microgels (Figure 7) showed similar results compared to the control gels (between 10 and 15% of whey separation). This high level of whey separation confirms the assumption that these aggregates exhibit only an inert role, unlike fractal aggregates or WP. However, their incorporation makes it possible to increase the protein concentration in acid milk gels without significantly altering their firmness properties. This was observed independently of the presence of fat.

The same type of results was obtained in the presence of mixed aggregates. Their addition did not lead to an increase in the storage modulus of the acid milk gels with AMF (Figure 6). Furthermore, the rheological analyses showed a slight decrease in the gelation pH compared to the control gel (shown for a concentration of 1% in Table 2). But, in the contrary of what occurred in the presence of microgels, mixed aggregates triggered a decrease in whey separation compared to control. Nevertheless, this was less effective than fractal aggregates or WP (Figure 7). They partially prevented the release of whey probably by decreasing the size of the gel pores or due to their water retention capacity. By using this type of aggregates, it became possible to

obtain acid milk gels with constant firmness and reduced whey separation even at the highest protein content. The consistency of gel modulus upon addition of mixed aggregates was quite surprising knowing that such aggregates are formed *in situ* via disulfide bridges during the heat treatment of conventional milk (Anema & Li, 2000; Haque & Kinsella, 1988; Jang & Swaisgood, 1990). In this case, mixed aggregates together with fractal aggregates induced an increase in gel strength (Donato & Guyomarc'h, 2009; Mahmoudi et al., 2011, 2010; Vasbinder et al., 2001). The addition of preformed mixed aggregates did not increase gel strength. They were probably inert due to the heat treatment used to produce the aggregates. This means that they were stable structures with different functionalities than the aggregates formed *in situ*. Moreover, these aggregates were not able to create connections with the protein network that forms during acidification.

3.2.4- Effect of the size of fractal aggregates and homogenization pressure at 3.5 and 10% AMF

The impact of different sizes of protein aggregates on the textural and microstructural properties of acidified milk model systems without fat has already been reported (Liu et al., 2016) and showed that smaller aggregates provide acid gels with higher firmness than larger aggregates. To evaluate the role of the interface or the one of the protein network on the strengthening of the acid dairy gels observed in the previous results, an increase in the interfacial surface was produced. This, in order to monitor if aggregates can modulate the texture of acid milk gels by an action at the interface.

Systems that contained 3.5 or 10% of anhydrous milk fat and fractal aggregates of different sizes were produced and homogenized at 100 or 500 bars before acidification. As previously stated, the mean diameter (D_{43}) of fat droplets was of approximately 0.8 μm in the systems containing 3.5% AMF that were homogenized at 100 bars. When these systems were homogenized at 500 bars, the mean diameter of fat droplets was of approximately 0.25 μm . The step of homogenization also impacts the diameter of fat droplets in recombined milks containing 10% AMF. The mean diameter of the droplets was of approximately 0.9 and 0.3 μm when the systems were homogenized at 100 and 500 bars, respectively. These conditions aimed to increase the total interfacial surface, to allow adsorption of other proteins than caseins at the interface. It has already been established that, with an identical fat concentration, emulsions containing small fat droplets reinforced the gels in a more pronounced way than emulsions with large fat droplets.

This reflected the importance of the number and sizes of the fat droplets in the gels (Xiong, Aguilera, & Kinsella, 1991).

The values of the final storage modulus obtained after 6 h of acidification in the presence of 1% fractal aggregates of different sizes are presented in Figure 8. Slightly higher values were obtained at 3.5% AMF and 100 bars in the presence of small fractal aggregates (Fa93 and Fa68). This result was also seen when recombined milk was homogenized at 500 bars, i.e. with a more developed interfacial surface. To further increase the interfacial surface, the same experiences were performed in the presence of 10% AMF. At 100 and 500 bars, the same trend was observed: an increased storage modulus was obtained when smaller fractal aggregates were used. This increase was more pronounced with a higher fat content (10%) and a high homogenization pressure, i.e. in the conditions where the interfacial surface was the highest. This change could be due to the development of higher number of interactions between the protein network and the aggregates because fractal aggregates of smaller size were in larger amount. The fact that no drastic change in the evolution of the moduli with the different aggregate was observed does not evidence a major role of the interface, except at 10% fat and 500 bars. The effect of decreasing aggregate sizes was mainly the same regardless of the fat content or the homogenization pressure. Under the tested conditions, this could mean that decreasing the size of the aggregates had mainly the same effect on the texture of gels, except in the case of the highest interfacial surface.

3.2.5- Confocal Scanning Laser Microscopy on acid milk gels

The microstructure of the acid gels containing 3.5% fat in the presence of 1% of various added proteins was observed (Figure 9) to visualize the protein network and the fat localization.

The control gel shows a sparse protein network (appearing in green), with large pores (in black). As a reminder, in this system, the rheological results gave a relatively weak elastic modulus and a significant whey separation was observed. The fat (in red) appeared well distributed within the gel and connected to the protein network.

The protein network in samples containing WP and fractal aggregates exhibited the same global aspect, but looked denser and more homogeneous than in the control. The pore sizes of the gels were seen decreased compared to control. This seems to support the higher results of elastic

modulus and the lower whey separation measured for these gels. The fat was well distributed within the gel and seemed connected to the protein network, as for the control. It has already been established that with a higher protein concentration, gel pores become smaller and a more homogeneous distribution of the protein material is observed. This leads therefore to a higher whey retention (Andoyo et al., 2014). The addition of whey protein had an impact on the establishment of the protein network and thus on the final texture of milk products. Fractal aggregates played the same role as WP. They seem encompassed in the protein network and contribute to its reinforcement.

The structure of the protein network containing microgel aggregates showed a greater heterogeneity than that of gels with WP or fractal aggregates, with proteins appearing more dispersed in the system. Larger whey zones compared to the gels with WP, quite similar to the structure of the control gel, were seen. This supports the rheological results which gave a final elastic modulus close to the value obtained for the control gel. Similar results were also obtained for whey separation. As the microgel aggregates were dense, they were less able to connect with the protein network.

The protein network with mixed aggregates also appeared visually more heterogeneous than when WP or fractal aggregates are added, but slightly more structured than with the addition of microgels. This evolution is the same than the one obtained for the whey separation results. Mixed aggregates appeared to be able to play a role, probably as inert filler, in the protein network, reducing the whey separation. The final elastic modulus obtained was close to the value of the control gel. The separate preparation of mixed aggregate and its further addition to milk did not lead to the same result as when they were formed *in situ*. This questions their structural difference in relation to their functionality. Even if it has been verified that the casein micelles used to manufacture the mixed aggregates were mostly in the micellar state, their fine structure could be different from the native one, hence impacting the functionality of the mixed aggregates.

These results are in agreement with those obtained by Liu et al. (2016). These authors have studied the impact of different types of protein aggregates of variable sizes on the textural and microstructural properties of acidified milk model systems without fat. The whey proteins were either nanoparticles ($< 1 \mu\text{m}$) or microparticles ($1 - 10 \mu\text{m}$). Nanoparticulated WP provided acid gels with higher firmness, faster gelation and lower whey separation coupled with a denser

microstructure. In contrast, microparticulated WP only weakly interacted with other proteins and their addition resulted in a protein network with low connectivity. Thus, the nature of the aggregates does not seem to be the only parameter to be taken into account. Their size also seems to be a key parameter in the modulation of the rheological properties of acid dairy gels. In our case, in the presence of fat, the same results were obtained: an improved texture, lower whey separation and more structured protein networks were obtained with the whey proteins and with the smaller fractal aggregates. Texture modulation by the protein aggregates seemed then to be mostly obtained by the protein interactions in the continuous phase and not by the connection of proteins from the droplet interfaces.

3.3- Interfacial composition

Although we aimed to modify and control the texture of acid milk gels through the addition of protein aggregates by connecting fat droplets, the results described above lead to the assumption that the observed modulation of the texture could mainly be due to an involvement of these aggregates in the continuous phase and not on the interface. To confirm this hypothesis, the interfacial composition of fat droplets in the milk before acidification was analyzed by electrophoresis.

Control milk and milks containing WP, fractal (Fa68) or mixed aggregates were prepared. The milks were enriched with 10% AMF and homogenized at 100 bars, to increase the interfacial surface. Unfortunately, due to experimental reasons, it was not possible to study systems homogenized at 500 bars. Indeed, the size of the fat droplets in these systems was too small. Milks and different phases of milk (3: containing the unabsorbed constituents and 5: containing desorbed proteins from the fat droplets) according to the Patton process as described above (see 2.4.2) were analyzed. Electrophoresis gels of milk and phase-5 and phase-3 control milks in non-reducing conditions, and phase-5 control milk and milks containing 1% WP, fractal or mixed aggregates in non-reducing and reducing conditions are presented in Figure 10.

The electrophoresis profile of total control milk in well call “milk” (Figure 10A) shows the presence of all the milk proteins. Bovine serum albumin (BSA), caseins, β -lactoglobulin and α -lactalbumin were found. For the emulsion’s aqueous phase (3), all milk proteins were also present. The main proteins which were found were caseins, but β -lactoglobulin and α -lactalbumin

were also present in this phase. In non-reducing conditions, protein aggregates that have not migrated were found at the top of the gel. These aggregates were found in the total control milk and aqueous phase (3) as well as in phase 5 which contained desorbed proteins from the fat droplets of the control milk. Compared to the other samples, phase 5 showed a less abundant content of protein aggregates and was mainly composed of caseins.

The aggregates found at the top of the electrophoresis gels were those that formed naturally during the heat treatment of milk. For systems containing added fractal and mixed aggregates (phase 5 in non-reducing condition), more aggregates at the top of the gel seemed to be found than for the control and system containing added WP. This would mean that some of the added preformed aggregates could have access to the interface of the fat droplets.

In phase 5, the electrophoresis profile in non-reducing conditions showed that these phases mostly contained caseins. Almost no β -lactoglobulin or α -lactalbumin was found in all the tested systems. It has been established that caseins preferentially adsorb to the surface of fat droplets compared to WP (Srinivasan, Singh, & Munro, 1996; Ye, 2008). If the casein concentration is sufficient, caseins can completely cover the fat droplet surface (Chevallier et al., 2016, 2018). However, in all systems, a small amount of aggregates could access the interface. This was confirmed by analyses of phase 5 in reducing conditions where bands matching β -lactoglobulin and α -lactalbumin were seen. These proteins were included in the composition of the aggregates present in the electrophoresis gels under non-reducing conditions and were probably also adsorbed on caseins. For phase 5 of milks with 1% fractal aggregates (Fa68) in reducing condition, more β -lactoglobulin and α -lactalbumin were observed than in other systems. It shows that these aggregates may have easier access to the interface.

It is therefore reasonable to claim that the interface of fat droplets in milk with 10% AMF and homogenized at 100 bars, was mainly covered by milk caseins. The additional whey proteins, added in the native form or as aggregates, rather played a role in the continuous phase. However, aggregates could be found at the interface in very small amounts, and this was more pronounced in presence of fractal aggregates. The increase of fat concentration to 10% and a homogenization pressure of 100 bars were not sufficient to allow the aggregates to reach the interface in large numbers and cause a large modification of interface connectivity. It should be noted that the same

experiments were performed on control milk containing 3.5% AMF and milk with added fractal aggregates (results not shown). Almost no aggregates were found in the phase containing desorbed proteins from the fat droplets. This confirms the hypothesis made from the rheological results that the whey proteins or the aggregates were mainly involved in the formation of the protein network. At 3.5% AMF, no difference was observed in the interfacial composition between the control milk and the milk with added fractal aggregates. It means that the interface of fat droplets was completely saturated with caseins.

4- Conclusions

This study has shown that adding different types of milk protein aggregates can change the texture of acid milk gels. The addition of whey proteins or fractal aggregates strengthened the structure of acid milk gels in the presence or in the absence of fat. Nevertheless, the addition of microgels or mixed aggregates had no impact on the rheological properties of acid milk gels.

The differences in textures obtained with the different types of aggregates with a low fat content (3.5%) were attributed to their involvement in the continuous phase of the systems. No significant impact of the aggregates on the composition of the fat droplet interface was evidenced, because of its saturation by the milk caseins. The different aggregates did not play the same role on the formation of the protein network: WP and fractal aggregates created a higher number of connections or stronger connections in the network which strengthened the protein network and reduced whey separation. Microgel and mixed aggregates behaved more like an inert material. Even if the acid dairy gel texture was not changed by adding such aggregates, mixed aggregates induced a decrease in whey separation. These types of aggregates allow for the formulation of protein-enriched acid dairy gels with controlled rheological properties.

The interfacial composition results showed that aggregates can access the interface when its surface is drastically increased. This limited access is due to the predominant ability of caseins to adsorb at interfaces. These results are in agreement with those found by Loiseleux (2017) who showed the ability of such aggregates to texturize emulsions containing 5% or 30% fat and very low concentrations of casein. Increasing fat content by over 10% or playing with the micellar caseins/WPN ratio or the fractal aggregates in milk acid gels could be a parameter to control the interfaces in such products and therefore could be used to modulate their texture. The difference

of functionality between preformed and *in situ* produced mixed and microgel aggregates should be further investigated.

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869

Preparation of reconstituted milk (with AMF)

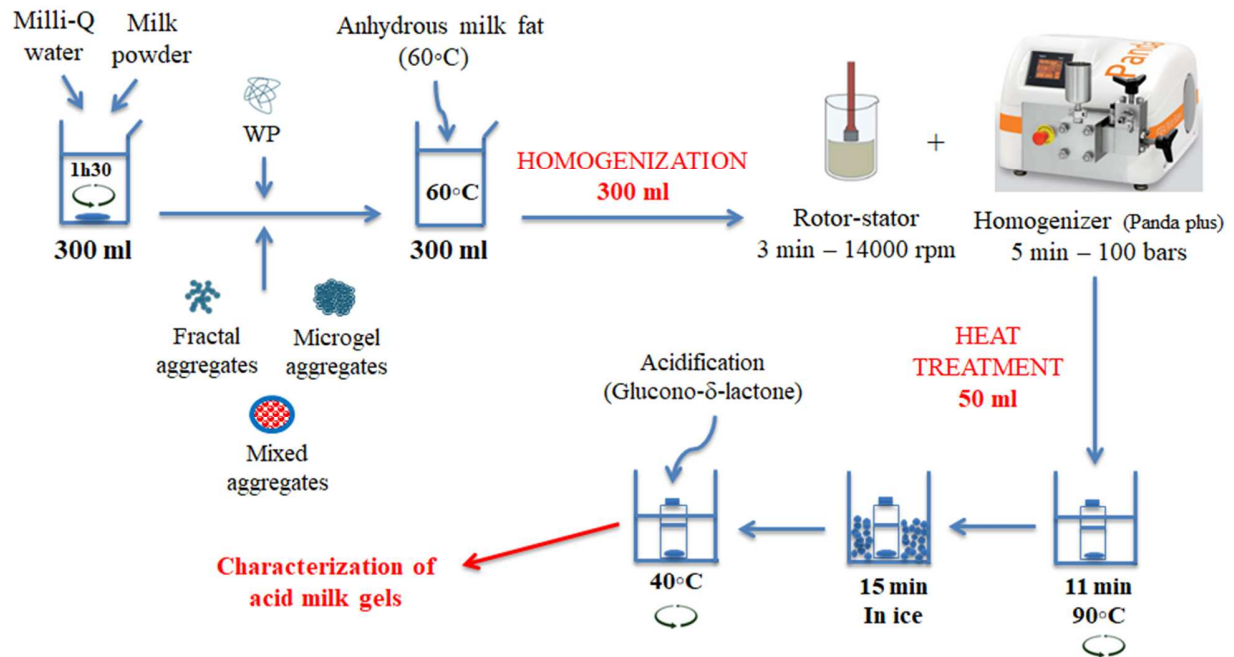


Figure 1: Experimental steps of preparation and acidification of reconstituted milk containing AMF and native whey proteins or protein aggregates

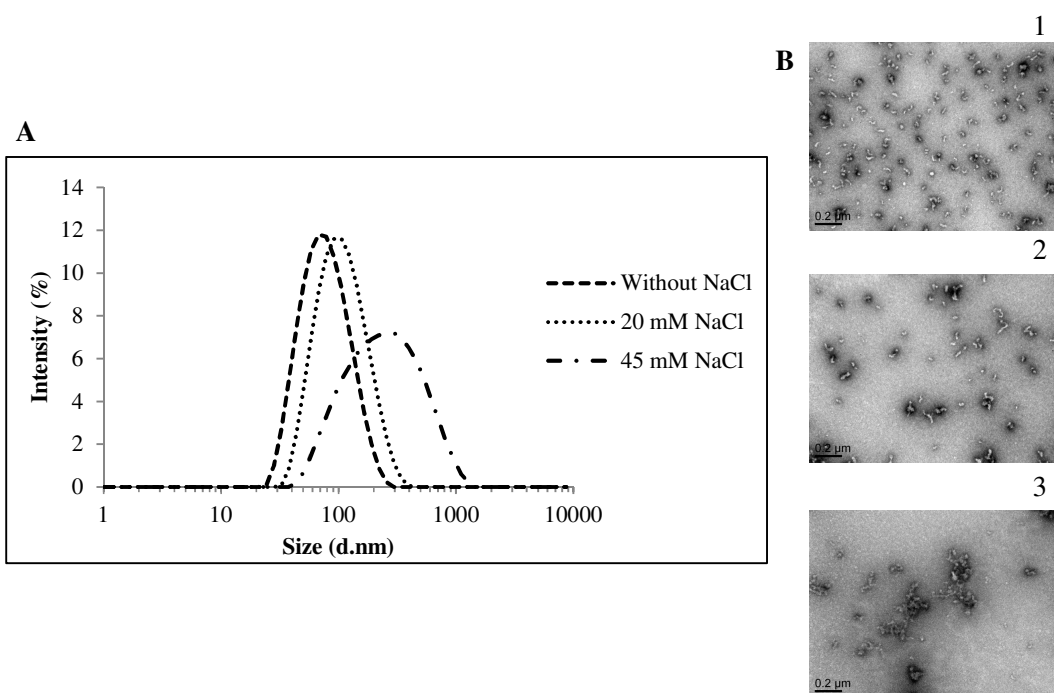


Figure 2: (A) Size distribution (DLS) of fractal aggregates obtained after heat treatment at 80 °C during 2 hours with variable concentrations of NaCl; (B) TEM micrographs (scale bar: 0.2 μ m) of fractal aggregates stained with 2% uranyl acetate (1: 0 mM, 2: 20 mM and 3: 45 mM NaCl)

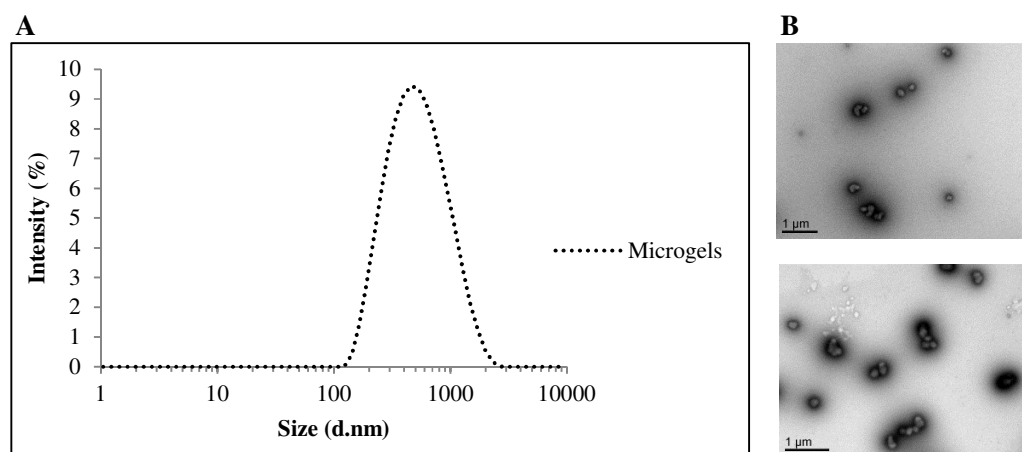


Figure 3: (A) Size distribution (DLS) of microgel aggregates obtained after heat treatment at 85 °C during 1 hour; (B) TEM micrographs of microgel aggregates stained with 2% uranyl acetate (scale bar: 1 μ m)

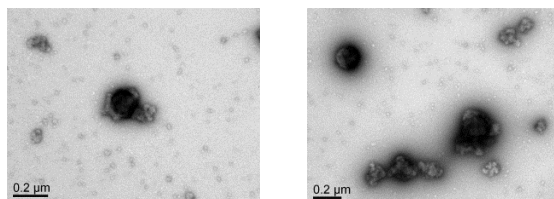


Figure 4: TEM micrographs of mixed aggregates obtained after heat treatment at 80 °C during 1 hour stained with 2% uranyl acetate (scale bar: 0.2 μm)

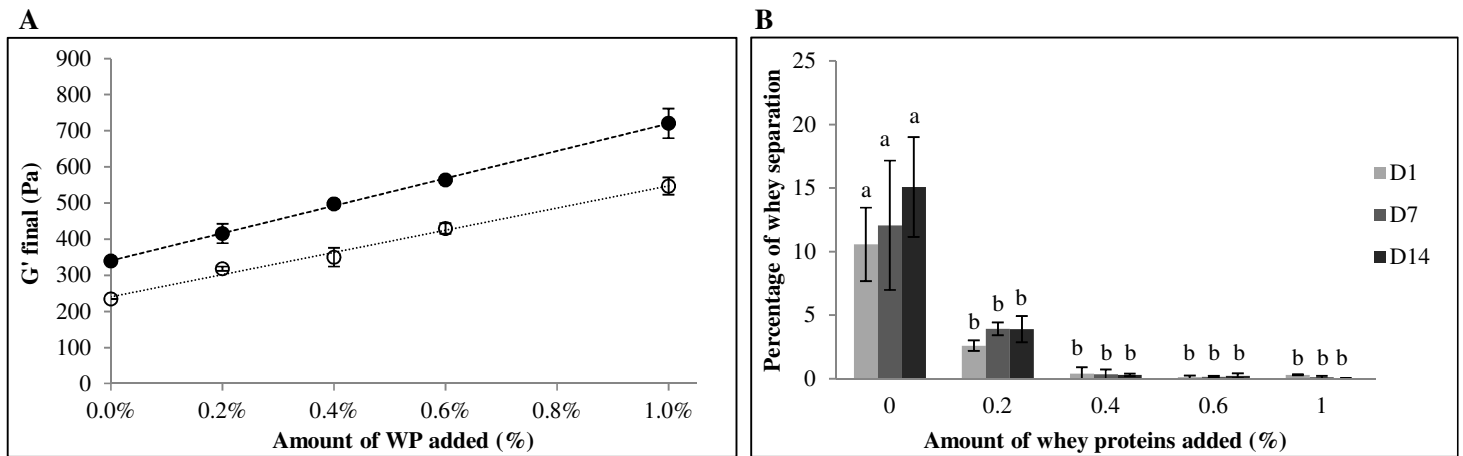


Figure 5: (A) Changes in the final storage modulus after 6 hours of acidification of acid milk gels with 3.5% AMF (black circle) or without AMF (empty circle) according to added WP quantity (0 to 1%); (B) Change of percentage of whey separation according to added WP quantity (0 to 1%) and time (day 1, 7 and 14) of gels at 3.5% AMF

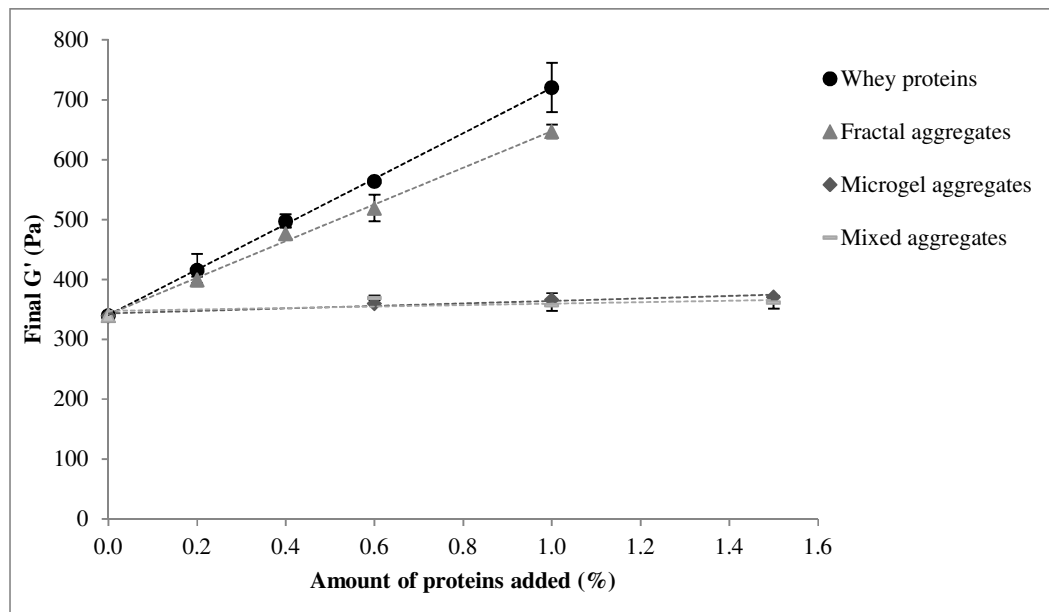


Figure 6: Change of the final storage moduli after 6 hours of acidification of acid milk gels (3.5% AMF) according to protein addition (whey proteins or fractal, microgel and mixed aggregates; 0 to 1.5%)

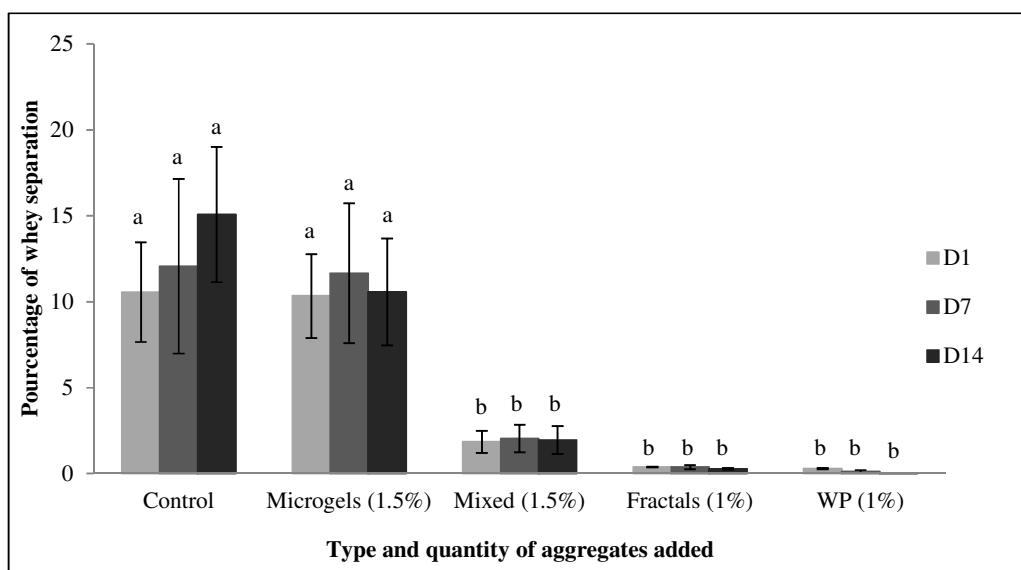


Figure 7: Change of percentage of whey separation according to type and quantity of added proteins (0 for control, 1% for fractals and WP and 1.5% for microgels and mixed aggregates) and time (days 1, 7 and 14)

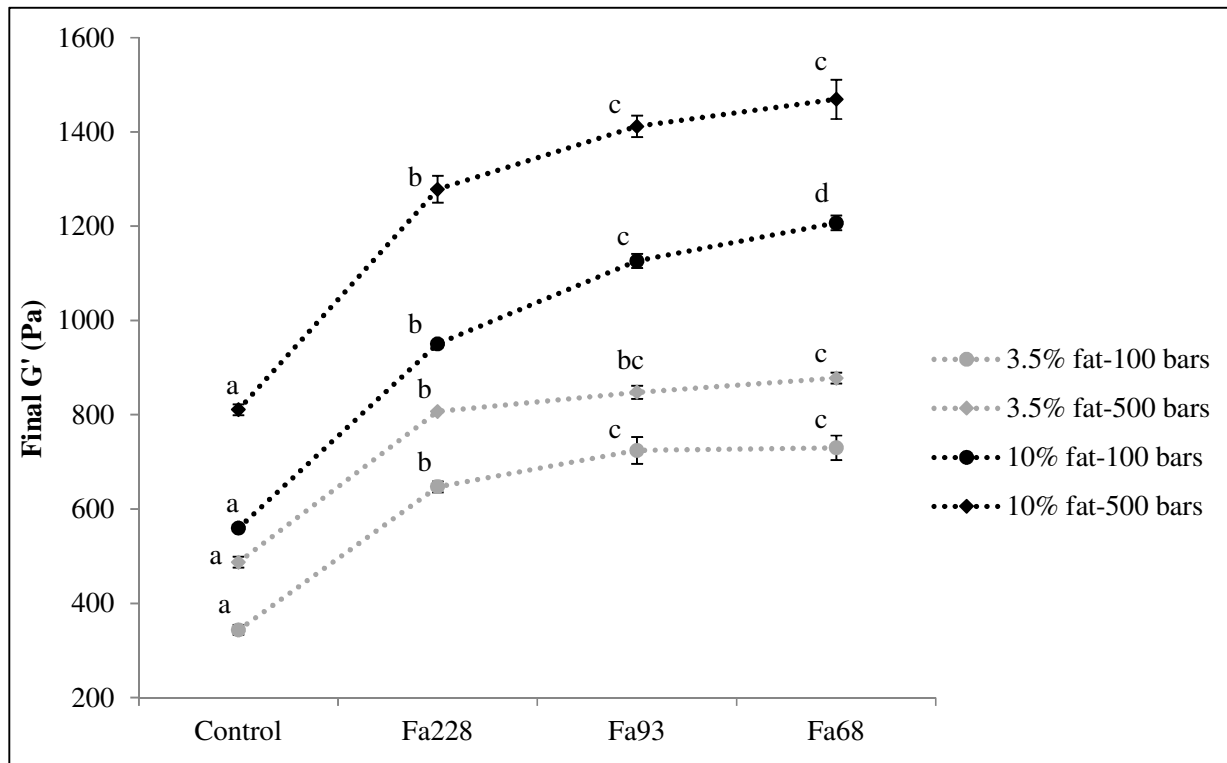


Figure 8: Storage modulus after 6 hours of acidification of control acid gels in presence of 1% fractal aggregates of different sizes (Fa228, Fa93 or Fa68) at two homogenization pressure (100 and 500 bars, 3.5% or 10% AMF)

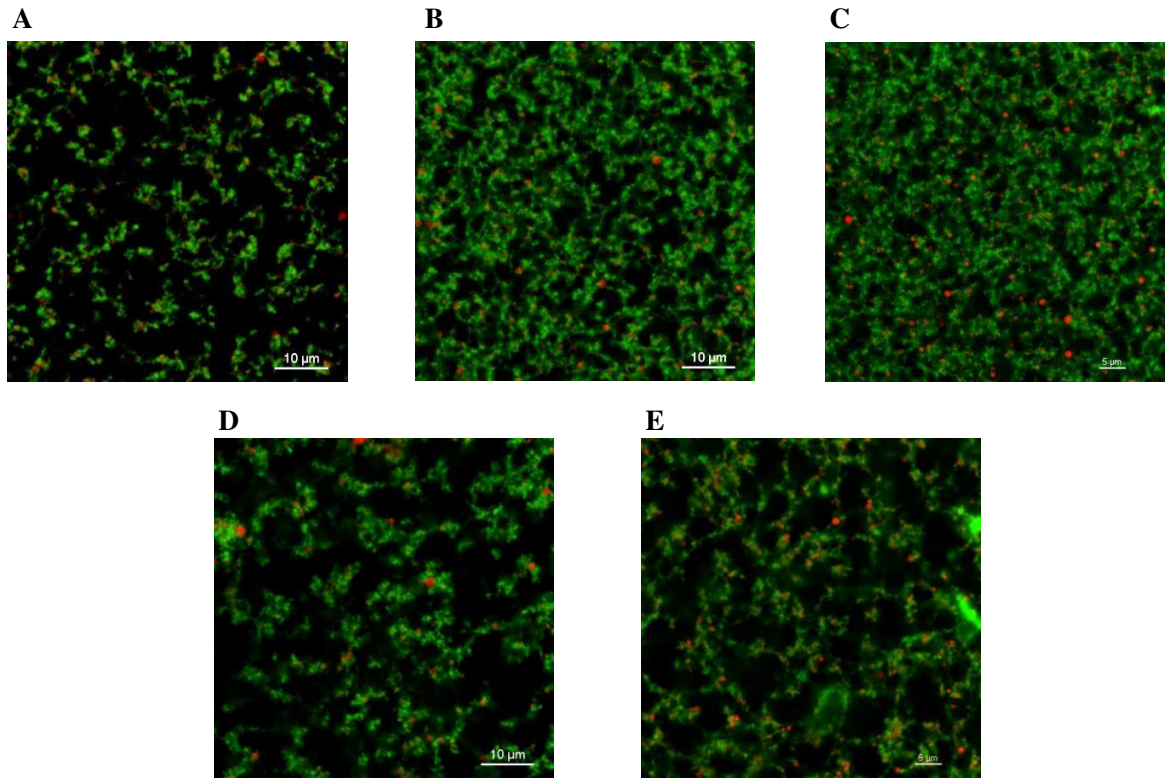


Figure 9: Confocal microscopy pictures of acid milk gels containing 3.5% AMF (A: control, B: 1% whey proteins, C: 1% fractal aggregates, D: 1% microgel aggregates and E: 1% mixed aggregates, homogenization pressure 100 bars) stained with Nile Red (for lipids) and Alexa 488 (for proteins)

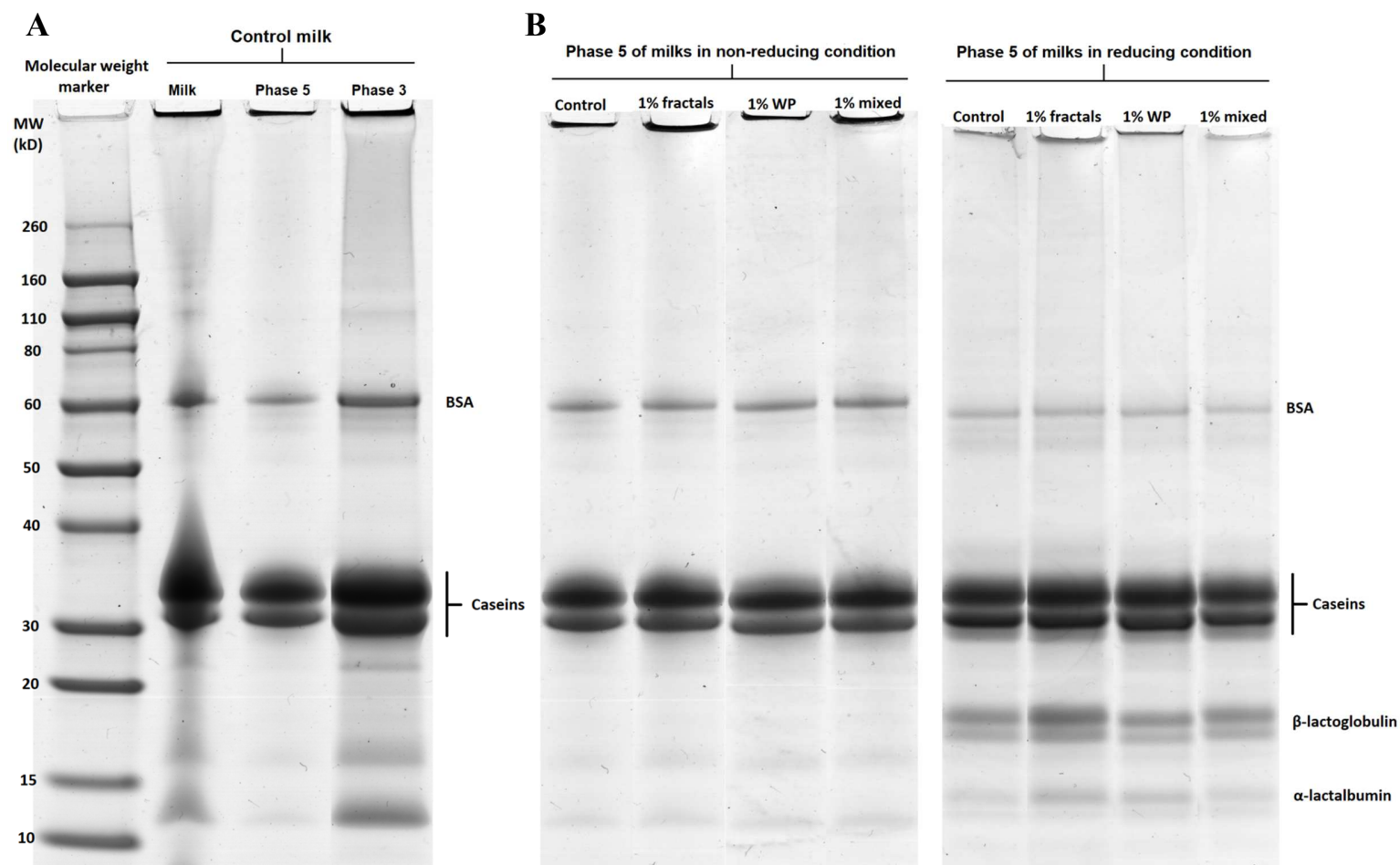


Figure 10: (A) SDS-PAGE of the interfacial composition of control milk with 10% AMF in non-reducing condition. (B) SDS-PAGE of the interfacial composition of phase 5 (containing the desorbed proteins from the interface) of control milk with 10% AMF and 1% fractals, WP or mixed aggregates in non-reducing and reducing conditions

Table 1: Low heat milk powder composition

Composition	Percentage
Humidity	3.4%
Fat	0.7%
Proteins/DDE* (Nx6.38)	38.6%
Lactose	56%
Ashes	8.5%
WPNI (<i>Whey Protein Nitrogen Index</i>)	≥ 6 mg/g

*DDE: defatted dry extract

Table 2: Average results and standard derivation for rheological and pH measurements on control acid milk gels without or with 3.5% AMF and acid milk gels with 1% added whey proteins or fractal, microgel or mixed aggregates (acidification 6h after GDL addition at 40 °C)

Sample	Gelation time (min)	Gelation pH	Tan δ amplitude	Final G' (Pa)	G' slope (FS)
Sample without AMF					
Control	29^c	5.44^a	0.52^d	234^a	0.164^d
Standard deviation	1.3	0.02	0	8.6	0.001
1% WP	22.4^{ab}	5.55^b	0.37^b	547^c	0.142^b
Standard deviation	0.2	0.01	0.01	24	0.003
1% fractals	19.6^a	5.62^b	0.33^a	390^b	0.129^a
Standard deviation	2.3	0.03	0.01	81	0.001
1% microgels	33.6^d	5.42^a	0.51^d	285^{ab}	0.162^{cd}
Standard deviation	0.1	0	0	6	0.001
1% mixed	27.3^{bc}	5.41^a	0.49^c	348^b	0.158^c
Standard deviation	0.1	0	0	9	0
Sample with 3.5% AMF					
Control	29.4^b	5.45^a	0.53^d	340^a	0.150^c
Standard deviation	2.3	0.03	0.04	6.4	0.003
1% WP	18.7^a	5.62^b	0.37^b	721^c	0.140^b
Standard deviation	0.1	0.01	0	41	0.001
1% fractals	20.7^a	5.60^b	0.35^a	647^b	0.133^a
Standard deviation	0.9	0.01	0.01	12	0.001
1% microgels	33.6^c	5.42^a	0.50^c	366^a	0.152^c
Standard deviation	0	0.01	0	11	0
1% mixed	31.4^{bc}	5.41^a	0.49^c	358^a	0.163^d
Standard deviation	0.8	0.02	0.01	10	0.002

Change of the texture of acid milk gel (3.5% AMF) according to protein aggregate type and concentration linked to their ultra-structure determined by confocal microscopy

